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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Process for the Inactivation of Viruses with the Aid of Acridine or Acridine Derivatives

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Process for the inactivation of viruses with the aid of acridine or acridine derivatives

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The invention relates to the use of acridine or acridine derivatives, preferably in combination with benzalkonium chloride, for the inactivation of enveloped or nonenveloped viruses. The process according to the invention is preferably carried out in the presence of proteins whose biological activity is largely retained.

It has been known for years that untreated human plasma or serum can contain human pathogenic viruses, such as HIV, HBV or HCV, which, if transmitted to sensitive recipients, can cause serious diseases, such as AIDS or hepatitis. In order to prevent this potential virus transmission, therapeutics which are obtained from human plasma or serum are prepared, on the one hand, only from preselected starting materials which, as far as anyone can judge, are virus-free and, on the other hand, are subjected to virus-inactivating/eliminating steps in the preparation process. The efficiency of the virus inactivation/elimination method used is established using strict measures and continuously checked.

Besides physical virus inactivation steps, chemical virus inactivation steps are also known in the preparation of said therapeutics. A particularly frequently discussed chemical process is the SD (solvent/detergent) method. It is suitable for inactivating enveloped viruses, i.e. viruses which are surrounded by a lipid-containing membrane, but has the crucial disadvantage of being completely ineffective against all known nonenveloped (uncoated) viruses. Moreover, also no other chemical process is known which would be suitable to inactivate

nonenveloped viruses while simultaneously retaining the biological activity of the protein constituents of the therapeutic or of the human plasma or serum.

Although the chemical virus inactivation processes are only used in a supplementary capacity to the physical methods, and although most viruses potentially transmissible by blood and blood products carry a lipid coat, for reasons of safety there is an exceptional need to make available chemical inactivation processes which also reliably inactivate nonenveloped viruses. This is all the more desirable as recently also HAV and parvoviruses, for example parvovirus B 19, were discussed as viruses which are potentially transmissible by blood fluids or blood products. (Vox Sanguinis 67, Supplement 1, 1994: Proceedings of a Symposium held at the New York Blood Center).

The invention was also based on the object of developing an industrially utilizable process for chemical virus inactivation, in which enveloped and nonenveloped viruses, e.g. parvoviruses, are inactivated with retention of the biological activity of proteins present, e.g. therapeutically useful proteins.

This object is achieved according to the invention by adding acridine or an acridine derivative to the protein-containing liquid to be treated. It was surprisingly found that acridine or acridine derivatives inactivate enveloped and nonenveloped viruses. Acridine derivatives are, for example, ethacridine, 9-aminoacridine (=aminacrine), 3,6-acridinediamine (proflavine), acrisorcin, Acrizane chloride (=phenacridane chloride), Acridine Orange, quinacrine, acricide, acridone, acridine-9-carboxylic acid, acranil (1-[(6-chloro-2-methoxy-9-acridinyl)amino]-3-(diethylamino)-2-propanol dihydrochloride), 3,7-diamino-5-phenylphenazinium chloride (phenosafranin, Safranin B Extra), phenoxazine, phenothiazine and especially acriflavine (3,6-diamino-10-methylacridinium chloride and 3,6-acridinediamine) and

their salts, e.g. chlorides, sulfates, bromides.

Surprisingly, it was additionally found that a combination of acridine or an acridine derivative and benzalkonium chloride displays a synergistic action during virus inactivation; i.e. the magnitude of the virus inactivation of the combination is higher than that of each individual substance.

The virus inactivation process according to the invention can be carried out with protein solutions such as blood, serum, plasma, blood products, allantoic fluid or milk. The virus inactivation is carried out at a pH of 3 to 10 or 5 to 9 (in, for example, serum or plasma solutions) and a temperature of 1°C to 80°C, preferably of 20°C to 60°C, very preferably from 20°C to 40°C, or 25°C to 37°C, and lasts 30 minutes to 10 hours, preferably 2 to 5 hours. For virus inactivation, a concentration of 1.0 g/l - 0.00001 g/l or 1.0 g/l - 0.004 g/l, preferably 0.1 g/l-0.001 g/l, is used for the acridines or acridine derivatives, and a concentration of 0.1 g/l - 0.004 g/l, preferably 0.05 g/l - 0.01 g/l, for benzalkonium chloride.

The removal of the acridines and of the benzalkonium chloride from the protein solutions, if this is necessary, is possible by means of simple, known methods, such as adsorption on active carbon or dialysis.

A further advantage of the virus inactivation process according to the invention is the very extensive protection of the protein constituents of the material to be treated: different biological activities, e.g. antibody activity and clotting activity, are not reduced or only reduced to a tolerable extent.

The virus inactivation process according to the invention can therefore be employed, for example, for the decontamination of the following materials:

- protein-containing solutions (dilute or concentrated)
- blood or blood products; both the liquid and the cellular constituents
- serum, plasma
- allantoic fluid
- organ extracts
- milk
- buffer solutions
- antigens for diagnostics
- vaccines, antigens for vaccines

The process according to the invention is furthermore suitable for disinfection in virus contamination of, for example, areas, equipment, effluents, wastes and surfaces of all types. The disinfection of virus-contaminated organ transplants, e.g. cornea, cerebral meninges, liver, heart, lungs or kidneys is also possible.

The present invention is furthermore illustrated by the following examples.

Methods generally used in the process according to the invention

Virus: Was replicated in a known manner in tissue cultures; the virus harvests were centrifuged and used as starting material for the further investigations.
The infectiousness titer was determined in microtiter plates - 8 replicates of 0.1 ml/- dilution stage - by double titration.

Stock solutions:	Ethacridine lactate (EL)	3% strength in dist. water)	
	Entozon® (E)	3% strength in dist. water}	Acridines
	Acriflavine (A)	1% strength in dist. water)	

Benzalkonium chloride (BACI)	10% in dist. water

General experimental procedure:
9 parts of buffer or medium or protein solutions were mixed with 1 part of virus. The addition of the amounts of the stock solutions indicated in the individual examples for virus inactivation and renewed mixing with subsequent virus titration were then carried out. After the times and temperatures mentioned in the individual examples, samples were removed and titrated in double determinations in order to be able to measure the process-related virus inactivation.

Types of virus investigated

Abbreviation	Name
PI_3V	Bovine parainfluenza 3 virus
BPV	Bovine parvovirus
PPV	Porcine parvovirus
IBRV	Infectious bovine rhinotracheitis virus
BVDV	Bovine viral diarrhoea virus
CPV	Canine parvovirus
BAV-1	Bovine adenovirus type 1
Reo 3	Reovirus type 3
Influenza A	Influenza A virus-Shangdong
Influenza B	Influenza B virus - B Panama

Further abbreviations/names used in the text:

EME Medium	Eagles Minimum Essential Medium
Beriate®P	Clotting factor VIII:C concentrate, pasteurized (Behringwerke AG, Marburg, Germany)

Haemate®p	Concentrate from clotting factor VIII and Von-Willebrand factor, pasteurized (Behringwerke AG, Marburg, Germany)
Beriplex®p	Pasteurized prothrombin complex concentrate (Behringwerke AG, Marburg, Germany)
Venimmun®	Human polyvalent immunoglobulin preparation (7S) for intravenous use (Behringwerke AG, Marburg, Germany)
Entozon®	Preparation of the following composition: 1 g of granules contains 0.059 g of dimethoxy-6-nitro-9-[(3-diethylamino-2-hydroxy)propylamino]acridine dihydrochloride and 0.295 g of ethacridine lactate (ASID Veterinär Vertriebs GmbH)
QAE cellulose	Diethyl-2-hydroxypropylaminoethyl cellulose (Ion exchanger for protein purification)
FCS	Fetal calf serum

Example 1

Inactivation of PI_3V by BACI

EME medium was mixed with PI_3 virus and BACI and incubated at 37°C in a water bath. After the times indicated, samples were taken to test for virus inactivation. The results are shown in Tab. 1.

Table 1

Inactivation of PI_3 virus with BACI at 37°C

Time (h)	BACI addition (mg/ml)	Virus titer found (log_{10}/ml)	Virus inactivation (log_{10}/ml)
0-0.03[1)]	0 (control)	7.4	-
	0.1	≤1.5	≥5.9
	0.005	≤1.5	≥5.9
	0.025	6.0	1.4
	0.0125	6.9	0.5
1	0 (control)	6.8	-
	0.1	≤1.5	≥5.3
	0.05	≤1.5	≥5.3
	0.025	≤1.5	≥5.3
	0.0125	5.5	1.3

[1)]Identical after addition of BACI and thorough mixing of the reaction batch

As emerges from the results in Table 1, PI_3 virus is very rapidly inactivated by the high doses of BACI (0.1 mg/ml, 0.05 mg/ml), i.e. in the time which was needed to mix the virus-containing sample thoroughly with BACI, to take a sample and to titrate this in order to determine the infectiousness of PI_3 virus, infectious virus was no longer detectable. At the two other BACI concentrations tested (0.025 mg/ml and 0.0125 mg/ml), a clear concentration-time dependence of virus inactivation is discernible.

Example 2

Virus inactivation by means of BACI or Entozon

The virus species shown in Tab. 2 were treated with BACI or Entozon and incubated at 45°C. Sample taking for virus titration was carried out 1 or 2 hours after the start of the test.

Table 2

Virus inactivation by means of BACI or Entozon at 45°C

Virus	Time	Substance Addition (mg/ml)	Virus titer found (log_{10}/ml)	Virus inactivation (log_{10}/ml)
BPV	1 hour	Control 0	4.7	0
		BACI 0.05	5.2	0
		" 0.01	5.2	0
	2 hours	Control 0	4.6	0
		BACI 0.05	4.6	0
		" 0.01	4.6	0
	1 hour	Control 0	4.7	0
		Entozon 0.030	≤1.5	≥3.2
		" 0.015	≤1.5	≥3.2
	2 hours	Control 0	4.6	0
		Entozon 0.030	≤1.5	≥3.1
		" 0.015	≤1.5	≥3.1
PPV	1 hour	Control 0	5.6	0
		Entozon 0.030	3.3	2.3
		" 0.015	3.7	1.9
	2 hours	Control 0	5.6	0
		Entozon 0.030	2.2	3.4
		" 0.015	2.7	2.9

As the results in Table 2 show, BPV is not inactivated by BACI under the test conditions selected. Inactivation is possible by means of Entozon, inactivation with BPV taking place substantially more rapidly than with PPV.

Example 3

Virus inactivation by means of BACI and acriflavine

The suspensions of the virus species shown in Tab. 3 were treated with BACI or acriflavine and incubated at 37°C for 2 hours. Samples were then taken for titration to determine the virus inactivation.

Table 3

Virus	Substance addition (mg/ml)		Virus titer found (log_{10}/ml)	Virus inactivation (log_{10}/ml)
IBRV	Control	0	5.1	0
	BACI	0.01	≤1.5	≥3.6
	Acriflavine	0.001	1.8	3.3
PI_3V	Control	0	5.6	0
	BACI	0.01	≤1.5	≥4.1
	Acriflavine	0.001	3.2	2.4
BVDV	Control	0	6.2	0
	BACI	0.01	2.3	3.9
	Acriflavine	0.001	2.5	3.7
BPV	Control	0	5.4	0
	BACI	0.01	5.9	0
	Acriflavine	0.001	≤1.5	≥3.9

As the results in Table 3 show, the enveloped virus species - IBRV, PI_3V, BVDV - are inactivated both by BACI and acriflavine, the inactivation by BACI being somewhat greater. The naked virus BPV is inactivated by acriflavine, but not by BACI alone.

After virus inactivation by means of acriflavine and BACI was detected in EME medium, it was checked whether these substances can also inactivate virus in protein-containing solutions. The following protein solutions were treated with the virus species indicated:

Beriplex®	PPV
Horse serum	BVDV, BPV
Beriate®	BVDV, BPV, BAV-1, Reo 3, IBRV
Haemate®	PPV, Reo 3
Venimmun®	BVDV, PPV, CPV
FCS	CPV
Egg allantoic fluid	Influenza A, Influenza B

The results obtained in these experiments are shown in tabular form below.

Example 4

Virus inactivation with acridines and benzalkonium chloride in protein solutions

As emerges from the results in Table 4, it is possible to inactivate completely different viruses in a methodologically simple manner in protein solutions using acridines and/or benzalkonium chloride. It appears clear here, however, that by means of benzalkonium chloride alone only envelope-containing viruses can be inactivated, whereas by means of acridines both envelope-containing and also nonenveloped virus species are inactivated. Both substances act synergistically in their virucidal action, i.e. the magnitude of the virus inactivation with the combination acriflavine + benzalkonium chloride is higher than with the two individual substances.

The results in Table 4 also show that virus inactivation in protein solutions is dependent on:

1. the virus species to be inactivated
2. the constituents and nature of the protein solution
3. the inactivating agent concentration
4. the inactivation time
5. the inactivation temperature.

The virus inactivation is also pH-dependent - results not shown. At a pH of below 5.5, virus inactivation takes place more slowly than at higher pHs.

Tables 5-7 contain the results of the biological activity of the protein solutions after virus inactivation by means of acriflavine and/or benzalkonium chloride. The biological activity was determined in Venimmun® by the content of antibodies before and after virus inactivation, and in Haemate®, Beriate® and Beriplex® by the determination of the clotting-promoting activity - measured in international units.

Table 4

Virus inactivation with acridines and benzalkonium chloride in protein solutions

Protein solution and virus	Inactivating agent	Concentration (mg/ml)	Inactivation Temperature (°C)	Inactivation time (h)	Control titer (log_{10}/ml)	Treatment titer (log_{10}/ml)	Titer reduction (log_{10}/ml)
Horse serum + BPV	Acriflavine	0.001	37	0 1 2	5.4 5.2 5.1	5.4 ≤1.5 ≤1.5	0 ≥3.7 ≥3.6
Horse serum + BVDV	Acriflavine	0.001	37	0 1 2	5.0 5.1 5.0	5.0 ≤1.5 ≤1.5	0 ≥3.6 ≥3.5
Beriate + BPV	Acriflavine	0.001	37	0 1 2	5.4 5.0 5.0	5.4 ≤1.5 ≤1.5	0 ≥3.5 ≥3.5
Beriate + BVDV	Acriflavine	0.001	37	0 1 2	5.0 5.0 4.8	5.0 ≤1.5 ≤1.5	0 ≥3.5 ≥3.5
Beriate + Reo 3	Ethacridine lactate	0.3	45	0 1 2	5.4 4.7 4.7	5.1 ≤1.5 ≤1.5	0.3 ≥3.2 ≥3.2
Beriate + IBR	Ethacridine lactate	0.3	45	0 1 2	6.5 5.7 4.3	6.5 2.0 ≤1.5	0 3.7 ≥2.8

Table 4 (continued)

Virus inactivation with acridines and benzalkonium chloride in protein solutions

Protein solution and virus	Inactivating agent	Concentration (mg/ml)	Inactivation Temperature (°C)	Inactivation time (h)	Control titer (log_{10}/ml)	Treatment titer (log_{10}/ml)	Titer reduction (log_{10}/ml)
Haemate + PPV	Acriflavine	0.001	37	0 1 2 3	5.4 5.5 5.5 5.3	5.5 3.3 1.9 ≤1.5	0 2.2 3.6 ≥3.8
Venimmun + PPV	Acriflavine	0.001	37	0 1 2	4.0 3.9 3.9	4.0 ≤1.5 ≤1.5	0 ≥2.4 ≥2.4
Venimmun + CPV	Acriflavine	0.001	37	0 1 2 3	6.9 6.8 6.8 6.9	6.6 4.8 3.9 3.9	0.5 2.0 2.9 3.0
Venimmun + BVDV	Acriflavine	0.001	37	0 1 2 3	6.4 5.8 6.3 6.0	6.4 4.5 1.9 0	0 1.3 4.4 ≥6.0

Table 4 (continued)

Virus inactivation with acridines and benzalkonium chloride in protein solutions

Protein solution and virus	Inactivating agent	Concentration (mg/ml)	Inactivation Temperature (°C)	Inactivation time (h)	Control titer (log_{10}/ml)	Treatment titer (log_{10}/ml)	Titer reduction (log_{10}/ml)
Egg allantoic fluid + Influenza virus B	Acriflavine	0.001	37	0 1 2	7.4 7.1 6.9	7.4 6.4 5.0	0 0.7 1.9
Egg allantoic fluid + Influenza virus B	BACI	0.02	37	0 1 2	7.4 7.1 6.9	7.4 6.0 3.6	0 1.1 3.3
Egg allantoic fluid + Influenza virus B	Acriflavine + BACI	0.001 ~ 0.02	37	0 1 2	7.4 7.1 6.9	7.4 4.1 2.5	0 3.0 4.4
Beriplex + PPV	Acriflavine	0.001	30	0 1 2 3	4.8 4.6 4.6 4.8	4.8 3.6 2.9 ≤1.5	0 1.0 1.7 ≥3.3
Beriplex + PPV	BACI	0.001	30	0 1 2 3	4.8 4.8 4.6 4.6	4.8 4.8 4.8 4.6	0 0 0 0
Beriate + PPV	Acriflavine + BACI	0.001 0.01	30	0 1 2 3	4.8 4.8 4.6 4.6	4.8 3.5 ≤1.5 ≤1.5	0 1.3 ≥3.1 ≥3.1

Table 4 (continued)

Virus inactivation with acridines and benzalkonium chloride in protein solutions

Protein solution and virus	Inactivating agent	Concentration (mg/ml)	Inactivation Temperature (°C)	Inactivation time (h)	Control titer (log_{10}/ml)	Treatment titer (log_{10}/ml)	Titer reduction (log_{10}/ml)
Beriplex + PPV	Acriflavine	0.001	37	0 1 2 3	4.8 4.6 4.6 4.8	4.8 2.0 ≤1.5 ≤1.5	0 2.6 ≥3.1 ≥3.3
Beriplex + PPV	BACI	0.01	37	0 1 2 3	4.8 4.6 4.6 4.8	4.8 4.6 4.6 4.6	0 0 0 0.2
Beriplex + PPV	Acriflavine + BACI	0.001 0.01	37	0 1 2 3	4.8 4.6 4.6 4.8	4.8 1.8 ≤1.5 ≤1.5	0 2.8 ≥3.1 ≥3.3
FCS + CPV	Acriflavine	0.001	37	0 1 2 3	6.6 n.d. n.d. 6.9	6.6 4.9 4.0 3.4	0 1.7 2.6 3.5
FCS + CPV	Acriflavine	0.001	45	0 1 2 3	6.8 n.d. n.d. 7.0	6.8 4.8 3.5 2.8	0 2.0 3.3 4.2

Table 5

Determination of the biological activity (antibody titer) in protein solutions before and after virus inactivation by means of acriflavine and benzalkonium chloride

Protein solution	Name	Temperature and duration of the treatment	Reciprocal neutralization titer[1] against polio virus Type 1	
			Without inactivating agent	With inactivating agent[2]
Low-cryo human plasma (individual plasma)	H1	37°C 2 hours	646	646
	H2		741	562
	H3		741	646
	H4		646	741
	H5		376	562
Venimmun® (Final product) various batches	V1	37°C 2 hours	2234	851
	V2		1950	1698
	V3		1698	1698
	V4		1698	1698
	V5		1479	1479
Low-cryo human plasma (individual plasma)	H1	45°C 2 hours	427	977
	H2		1288	977
	H3		1288	977
	H4		977	1122
	H5		977	1288
Venimmun® (Final product) various batches	V1	45°C 2 hours	1950	2234
	V2		2570	2234
	V3		2234	1479
	V4		2234	1950
	V5		2234	1479

[1] Titer calculation according to Spearman-Kärber from $\log_2$ dilution series using 8 replicates/dilution stage

[2] Acriflavine 0.001 mg/ml + BACI 0.02 mg/ml

Table 6

Determination of the biological activity (antibody titer) in protein solutions before and after virus inactivation by means of acriflavine and benzalkonium chloride

Protein solution	Name	Temperature and duration of the treatment	Rec. HAH anti-PI, virus Antibody titer[1]		Rec. HAH anti-reo3 virus Antibody titer[1]	
			Without I.[2]	With I.[2]	Without I.[2]	With I.[2]
Low-cryo human plasma (individual plasma)	H1	45°C 2 hours	80	80	320	320
	H2		80	80	320	320
	H3		80	80	320	320
	H4		80	80	320	320
	H5		80	80	320	320
Venimmun® (Final product) various batches	V1	45°C 2 hours	320	320	320	320
	V2		320	320	320	320
	V3		320	320	320	320
	V4		320	320	320	320
	V5		320	320	320	320
Horsye serum (individual sera)	P1	45°C 2 hours	n.d.	n.d.	640	640
	P2				640	640
	P3				640	640
	P4				640	640
	P5				640	640

[1] Titer determination in $\log_2$ dilution series

[2] I. = inactivating agents: acriflavine 0.001 mg/ml + BACI 0.02 mg/ml

Table 7

Determination of the biological activity (clotting activity) of protein solutions before and after virus inactivation by means of acriflavine (A) and benzalkonium chloride (BACL)

Protein solution	Temperature and duration of the treatment	Inactivating agent	Inactivating agent concentration (mg/ml)	Activity (IU/ml)
cryo-protein solution after $Al(OH)_3$ + QAE treatment	3 hours 45°C	A	0.01	3.9
		A	0.03	4.2
		A	0.001	4.4
		BACI	0.1	6.9
		BACI	0.05	6.1
		A + BACI	0.001 + 0.05	4.1
		Control	0	5.3
Haemate® Factor VIII	3 hours 37°C	A	0.002	18.9
		A	0.001	16.8
		BACI	0.02	20.7
		A + BACI	0.001 + 0.02	17.6
		Control	0	24.1
Beriplex® Factor II	3 hours 37°C	A	0.002	48.8
		A	0.001	50.0
		BACI	0.02	61.3
		A + BACI	0.001 + 0.02	47.9
		Control	0	68.6

As emerges from the results in Tables 5 and 6, the content of neutralizing (Polio Type 1) and hemagglutination-inhibiting (HAH) antibodies (PI_3- and Reo 3 virus) in Venimmun® is not

affected to an extent exceeding the test variations (as a rule ± 1 $\log_2$ stage) during virus inactivation by means of acridines and benzalkonium chloride. The activity decrease with the clotting preparations (Beriate®, Haemate®, Beriplex®) after acridines/benzalkonium chloride inactivation is tolerable (Table 7).

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for the inactivation of viruses, which comprises employing acridine or an acridine derivative.

2. The process as claimed in claim 1, wherein benzalkonium chloride is additionally employed.

3. The process as claimed in claim 1 or 2, which is carried out in the presence of proteins and wherein the biological activity of these proteins is retained.

4. The process as claimed in claim 1, 2 or 3, wherein the incubation is carried out at a temperature of 20-60°C.

5. The process as claimed in one of the preceding claims 1-4, wherein the temperature is 25-37°C.

6. The process as claimed in one of the preceding claims 1-5, wherein the incubation is carried out at pH 5-9.

7. The process as claimed in one of the preceding claims 1-6, wherein acridine or the acridine derivative is employed in a concentration of 0.0001 to 1.0 g/l.

8. The process as claimed in claim 7, wherein acridine or the acridine derivative is employed in a concentration of 0.005 to 0.1 g/l.

9. The process as claimed in one of the preceding claims 2-8, wherein benzalkonium chloride is employed in a concentration of 0.004 to 0.1 g/l.

10. The process as claimed in claim 9, wherein benzalkonium chloride is employed in a concentration of 0.001 to 0.05 g/l.

11. The process as claimed in one of the preceding claims 1-10, wherein the incubation time is 0.5 h - 10.0 h.

12. The process as claimed in claim 11, wherein the incubation time is 2-5 h.

13. The process as claimed in one of the preceding claims 1-12, wherein the viruses are parvoviruses or other nonenveloped viruses.

14. The process as claimed in one of the preceding claims 1-13, wherein the viruses are enveloped viruses.

2164766

BEHRINGWERKE AKTIENGESELLSCHAFT 1994/B020 - Ma 1043

Abstract

Process for the inactivation of viruses with the aid of acridine or acridine derivatives

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The invention relates to the use of acridine or acridine derivatives, preferably in combination with benzalkonium chloride, for the inactivation of enveloped or nonenveloped viruses. The process according to the invention is preferably carried out in the presence of proteins whose biological activity is substantially retained.